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Cloning the Full-Length cDNA for Rat Connective Tissue Growth Factor: Implications for Skeletal Development

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Abstract The mammalian osteopetroses represent a pathogenetically diverse group of skeletal disorders characterized by excess bone mass resulting from reduced osteoclastic bone resorption. Abnormalities involving osteoblast function and skeletal development have also been reported in many forms of the disease. In this study, we used the rat mutation, *osteopetrosis* (*op*), to examine differences in skeletal gene expression between *op* mutants and their normal littermates. RNA isolated from calvaria and long bones was used as a template for mRNA-differential display. Sequence information for one of the many cDNA that were selectively expressed in either normal or mutant bone suggested that it is the rat homologue of connective tissue growth factor (CTGF) previously cloned in the human, mouse, and other species. A consensus sequence was assembled from overlapping 5′-RACE clones and used to confirm the rat CTGF cDNA protein coding region. Northern blot analysis confirmed that this message was highly (8- to 10-fold) over-expressed in *op* versus normal bone; it was also upregulated in *op* kidney but none of the other tissues (brain, liver, spleen, thymus) examined. In primary rat osteoblast cultures, the CTGF message exhibits a temporal pattern of expression dependent on their state of differentiation. Furthermore, CTGF expression is regulated by prostaglandin E_2, a factor known to modulate osteoblast differentiation. Since members of the CTGF family regulate the expression of specific genes, such as collagen and fibronectin, we propose that CTGF may play a previously unreported role in normal skeletal modeling/remodeling. Its dramatic over-expression in the *op* mutant skeleton may be secondary to the uncoupling of bone resorption and bone formation resulting in dysregulation of osteoblast gene expression and function. J. Cell. Biochem. 77:103–115, 2000.

Key words: connective tissue growth factor; osteopetrosis; osteoblast development

The formation and maintenance of the vertebrate skeleton require the interactions of many cell types, and the past decade has witnessed an explosive growth in our understanding of growth factors and other molecules that mediate the complex coordination of bone formation and bone resorption in skeletal modeling and remodeling [Popoff and Marks, 1997]. This article describes the expression of a previously reported growth factor, connective tissue growth factor (CTGF), in a new tissue, the skeleton, where we found this gene to be highly upregulated in an animal model of osteopetrosis, the *osteopetrosis* (*op*) mutation in the rat.

CTGF is a cysteine-rich peptide first discovered by Bradham and colleagues [1991] by screening a human umbilical vein endothelial cell cDNA expression library using a polyclonal anti-PDGF antibody. At about the same time, two independent groups isolated mouse CTGF (FISP 12/βIG-M2) from serum-stimulated NIH-3T3 cells and TGF-β-stimulated mouse AKR-2B cells, using differential cloning techniques [Ryseck et al., 1991; Brunner et al., 1991]. CTGF has since been isolated, cloned, and sequenced in other species, including the cow [Lin et al., 1998], pig [Brigstock et al., 1997], and frog [Ying and King, 1996] but, to date, it has not

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been cloned in the rat. The CTGF gene belongs to a larger CCN gene family that also includes Cyr61 [O'Brien et al., 1990]/CEF10 [Simmons et al., 1989] and *nov* [Joliot et al., 1992]. All members of the CTGF gene family exhibit a high degree of amino acid sequence homology (50–90%), possess a secretory signal peptide at the N-terminus, and contain 38 conserved cysteine residues [Oemar and Lüscher, 1997]. This family has four distinct protein modules, which include (1) an IGF-binding domain, (2) a von Willebrand factor type C repeat, (3) a thrombospondin type I repeat, and (4) a C-terminal module [Oemar and Lüscher, 1997].

With the exception of *nov*, CTGF family members are immediate early growth-responsive genes that are believed to regulate the proliferation/differentiation of various connective tissue cell types [Joliot et al., 1992; Kothapalli et al., 1998]. It has been postulated that CTGF family members play a role in various processes including embryogenesis, wound healing and extracellular matrix production in various connective tissues [Surveyor et al.,1998; Frazier et al., 1996]. Over-expression of CTGF has also been implicated in the pathogenesis of numerous sclerosing diseases such as renal fibrosis [Ito et al., 1998], inflammatory bowel disease (e.g., Crohn's disease and ulcerative colitis) [Dammeier et al., 1998], atherosclerosis [Oemar et al., 1997], and scleroderma [Igarashi et al., 1996].

CTGF mRNA is expressed in many tissues with highest levels in the kidney and brain [Ryseck et al., 1991; Oemar and Lüscher, 1997]. To date, CTGF mRNA expression or protein production has been demonstrated in endothelial cells [Lin et al., 1998], fibroblasts [Dammeier et al., 1998; Kireeva et al., 1997] and chondrocytes [Nakanishi et al., 1997]. CTGF is believed to act as an autocrine or paracrine regulator of cell proliferation, migration and/or adhesion, having site-specific effects dependent on the target cells [Kireeva et al., 1997]. It clearly promotes proliferation and extracellular matrix production in fibroblasts and is believed to regulate angiogenesis via its effects on endothelial cells [Oemar and Lüscher, 1997]. Nakanishi and colleagues [1997] recently showed by in situ hybridization that CTGF was selectively expressed in hypertrophic chondrocytes within growth plate cartilage suggesting that CTGF may play a role in endochondral ossification. In addition, it has been shown that CTGF gene expression is induced by TGF-β indicating that it is downstream of the TGF-β-induced signaling pathway [Grotendorst, 1997].

In this study, we used the *osteopetrotic* (*op*) rat as a model to examine differential gene expression in bone from normal and osteopetrotic bone. Osteopetrosis describes a group of congenital bone disorders that are characterized by a generalized increase in skeletal mass resulting from a primary defect in osteoclast-mediated bone resorption [Popoff and Schneider, 1996]. Numerous osteopetrotic mutations have been described in several species, including human, mouse and rat [Popoff and Schneider, 1996]. The bone formed as the skeleton develops and grows is not resorbed, resulting in the failure to develop bone marrow cavities. The osteopetrotic mutations are pathogenetically heterogeneous, as the point at which osteoclast development or activation is intercepted differs for each mutation [Popoff and Marks, 1995]. Although osteoclast hypofunction is universal among the osteopetrotic mutations, abnormalities involving osteoblast development/function (i.e., bone formation), mineral homeostasis, and the immune and endocrine systems have also been reported [Seifert et al., 1993]. The *op* rat mutation was selected for this study because of its severe skeletal phenotype [Marks and Popoff, 1989]. A comparison of gene expression in normal versus *op* long bones and calvaria using mRNA-differential display resulted in the identification and cloning of rat CTGF. CTGF mRNA is highly over-expressed in *op* versus normal bone. In addition to being the first report of rat CTGF nucleotide and predicted amino acid sequence, these studies also provide evidence that this growth factor plays a role in osteoblast development. The implications of these findings with respect to the development and maintenance of bone in both physiological and pathological conditions are discussed.

MATERIALS AND METHODS

Source of Animals

An inbred colony for the *osteopetrotic* (*op*) mutation in the rat, consisting of heterozygous breeders (+/*op*), is maintained at Temple University School of Medicine. Mutants (*op*/*op*) and normal littermates (+/?) were distinguished radiographically 1–3 days after birth by the failure of development of marrow spaces in mutants [Schneider et al., 1979]. Because the genotype of phenotypically normal rats cannot be distinguished except by breeding experiments, the normal littermates used in this study

were of either heterozygous (+/*op*) or homozygous (+/+) normal genotype. All animals were maintained and used according to the principles in the NIH Guide for the Care and Use of Laboratory Animals [1985], and guidelines established by the IACUC of Temple University.

Primary Osteoblast Cultures

Normal diploid osteoblasts were isolated from the calvaria of 21-day gestation fetal rats by sequential trypsin/collagenase digestion and plated in 100-mm dishes in minimum essential medium (MEMα)(Gibco-BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts, Calabas, CA) and 1% Penicillin-Streptomycin (P-S) (Gibco-BRL Life Technologies) at a density of 7.5×10^5 cells/dish [Owen et al., 1990]. Media was changed every other day throughout the time course of culture and for media changes after day 6 of cultures, MEMα supplemented with 50 μg/ml ascorbic acid, 2 mM inorganic phosphate, 10% FBS, and 1% P-S was used to feed the cells. For prostaglandin E_2 (PGE_2) treatment, PGE_2 dissolved in ethanol was added to the media at plating and at every feeding to a final concentration of 10^{-6} M and cells were harvested 48 h after feeding on the indicated days. Vehicle-treated cells received a final concentration of 0.01% ethanol.

RNA Isolation

Total cellular RNA was isolated from calvaria and long bones (femurs and tibias) harvested from 2-week-old mutant and normal rats of *op* stock. The calvaria and long bones were cleaned free of soft tissue, flash-frozen in liquid nitrogen, and stored at −80°C. Before freezing, the ends of the long bones were removed at the growth plate, and bone marrow was flushed from the shafts of normal bones with saline (4°C), using a 25-gauge needle. Flushing of the bone marrow was only possible in normal rats because there were no marrow cavities in *op* mutants. Total RNA was prepared as previously described [Thiede et al., 1994]. Briefly, a minimum of six samples per phenotype and bone site (calvaria versus long bone) were pooled and used to prepare a bone powder by rapidly pulverizing frozen samples in a Bessman tissue pulverizer (Fisher Scientific, Pittsburgh, PA) precooled in a bath of dry ice/ethanol. Bone powder was homogenized in an RNA extraction buffer consisting of 5 M guanidinium-isothiocyanate, 72 mM β-mercaptoethanol and 0.5% Sarkosyl. Homogenates were layered over a 3.0-ml CsCl cushion (5.7 M CsCl and 30 mM NaAc) and centrifuged at $100{,}000 g_{av}$ overnight (14–16 h) at 20°C. RNA was recovered as a translucent pellet after centrifugation. Total RNA was isolated from the primary osteoblast cultures by the same procedure without pulverizing. RNA was isolated from kidney, liver, spleen, thymus, and brain harvested from 2-week-old *op* rats and their normal littermates, using TRIzol (Gibco-BRL Life Technologies). The RNA concentration of each sample was quantitated by absorbance at 260 nm. The integrity and accuracy of the spectrophotometric measurement of each RNA sample were assessed by electrophoresis of 1 μg on an ethidium bromide-stained, formaldehyde-agarose minigel.

Differential Display of mRNA

Before differential display, bone RNA samples were treated with DNase I (Boehringer Mannheim, Indianapolis, IN) to eliminate any potential contamination with genomic DNA. The basic principle of mRNA differential display was first described by Liang and Pardee [1992]. Briefly, 0.5 μg RNA from each sample (total of four independent samples, mutant and normal/calvaria and long bone) was reverse-transcribed using each of 12 two-base-anchored oligo-dT primers provided in the Hieroglyph mRNA profile kits (Beckman Coulter, Fullerton, CA) to subdivide the mRNA population. First-strand cDNA were amplified by the polymerase chain reaction (PCR) for 30 cycles, using one of 4 upstream arbitrary primers (also provided in the kit) and the same anchoring primers used for first-strand synthesis. This resulted in 48 possible primer combinations for each kit (total of 5 kits); each PCR amplification was run in duplicate from the same first-strand cDNA template. All amplified cDNA were radiolabeled with ^{33}P-dATP. The radiolabeled PCR products were electrophoresed on 4.5% denaturing polyacrylamide gels and dried using the Genomyx LR differential display apparatus (Beckman Coulter). After autoradiography, bands were visually assessed; those representing differentially expressed cDNA (exclusively expressed or highly overexpressed in one phenotype and confirmed in duplicate PCR amplification) were excised from the gel. Each cDNA of interest was reamplified by PCR and used to probe a Northern blot to confirm its differential expression.

Northern Blot Analysis

Twenty µg of total RNA from *op* mutant and normal bone/soft tissue or normal osteoblast cultures was electrophoresed on 1% formaldehyde-agarose gels and transferred to nylon membranes (Scheicher & Schuell, Keene, NH). Blots were hybridized with random-prime labeled ([α-^{32}P]dCTP, 6,000 Ci/mmol, Amersham, Arlington Heights, IL) rat CTGF, rat alkaline phosphatase [Noda et al., 1987], or rat osteocalcin [Lian et al., 1989] probes (Rediprime™ Amersham Pharmacia Biotech, Piscataway, NJ). Two CTGF probes were used, including 717 bp of the 3′ untranslated region and 1,200 bp of the coding region; both probes generated identical results. Blots were then autoradiographed, stripped, and re-probed with an 18S cDNA probe used as a control to normalize for differences in loading and transfer. Each autoradiograph was digitized and band intensities were quantitated using SigmaGel (Jandel Scientific, San Rafael, CA) analysis software.

Cloning and Restriction Analysis of Individual cDNA

In many cases, the Northern confirmation of cDNA extracted from differential display gels revealed multiple bands. To isolate individual cDNA and obtain the cDNA of interest, cDNA were cloned into the PCR-Script vector (PCR-Script™ Amp Cloning Kit, Stratagene, La Jolla, CA) and transformed into *Escherichia coli* by electroporation. Transformed bacteria were plated and incubated at 37°C overnight. Individual colonies (up to 20) were randomly chosen and placed in 2 ml LB medium with 50 µg/ml Ampicillin for overnight incubation at 37°C. A small aliquot (20 µl) from each plasmid preparation was lysed, and the KS and T3 primers flanking both sides of the target inserts were used for polymerase chain reaction (PCR). A small amount (10 µl) of the PCR product was electrophoresed on a 1.5% agarose gel to check for the correct size using a 100-bp DNA ladder (Promega, Madison, WI). The remaining PCR product was purified (QIAquick PCR purification kit, Qiagen, Valencia, CA) and used for a restriction enzyme analysis using *Hpa*II and *Rsa*I. Depending on the number of restriction patterns obtained, a plasmid preparation corresponding to each pattern was used to prepare a labeled cDNA probe for subsequent Northern confirmation. Individual cDNA of interest were then sequenced.

Cloning of Rat CTGF cDNA

Approximately 717 bp of sequence corresponding to the 3′ end of rat CTGF was obtained from differential display. In order to generate additional sequence data, 5′ RACE was performed using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Two nested CTGF specific primers were used to increase reaction specificity and multiple independent reactions were performed to decrease the incidence of sequence errors introduced by the PCR reactions. 5′ RACE products were sequenced, aligned with the sequence obtained from the differential display clone, and a consensus sequence was developed. PCR primers were then designed based on this consensus sequence, and the rat CTGF cDNA was amplified from RNA isolated from *op/op* mutant rat calvaria.

DNA Sequencing

DNA was sequenced using standard dideoxy methodologies. Gaps and ambiguities in the sequence were handled by direct sequencing of required regions using specific primers.

RESULTS

RNA prepared from calvaria and long bones of 2-week-old osteopetrotic (*op*) and normal rats was used as a template for differential display (DD). Many differences in gene expression were evident between mutant and normal bone, as can be seen in the autoradiogram shown in Figure 1. For CTGF, the upper of the two intense bands in mutant calvaria and long bone that is faintly visible in normal bone represents the band from which the original CTGF fragment was isolated (Fig. 1). When this band was cut from the gel, reamplified, and used to probe confirmatory Northern blots, there were several distinct bands visible (data not shown), but only one of these demonstrated differential gene expression similar to that in the original DD autoradiogram. Because this was likely due to the presence of more than one cDNA extracted from the DD gel, the re-amplified band was cloned, grown in *E. coli*, and individual clones were analyzed by restriction digestion.

Ethidium bromide-stained agarose gels displayed five different restriction patterns (data not shown). A corresponding cDNA representing each of the five patterns was radiolabeled and used to re-probe Northern blots containing

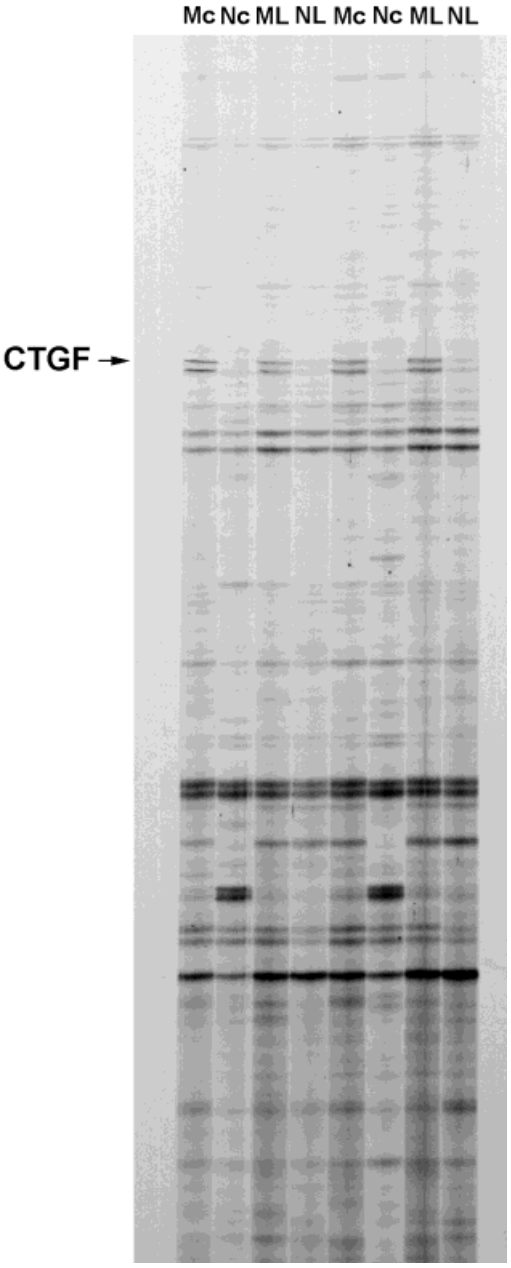

Fig. 1. Representative autoradiograph from a differential display gel. Total cellular RNA obtained from mutant (M) or normal (N) calvaria (c) and long bone (L) was reverse-transcribed using a two-base-anchored oligo-dT primer (Hieroglyph mRNA profile kit). First-strand cDNA was used to generate radiolabeled polymerase chain reaction (PCR) products using the same anchoring primer and an arbitrary upstream primer (also provided in the kit). Each PCR reaction was run in duplicate and displayed side by side on a sequencing type gel. The arrow points to the band that contained the CTGF cDNA showing a consistent and substantial difference in expression between mutant versus normal bone. The size of the cDNA isolated from this band were approximately 700 bp. Additional differences can be seen, some of which are currently under investigation.

RNA from normal and mutant bone. Each probe hybridized to yield a single band that corresponded to one of the multiple bands from the original Northern blot. One of these (originally named clone 43) proved to be the cDNA of interest, being highly (8- to 10-fold) overexpressed in *op* compared with normal bone (Fig. 2). This 717 bp cDNA was sequenced and demonstrated considerable homology with human CTGF and mouse FISP12 although the entire fragment was in the 3′ UTR.

This cDNA was used to generate additional 5′ RACE clones. A consensus sequence was

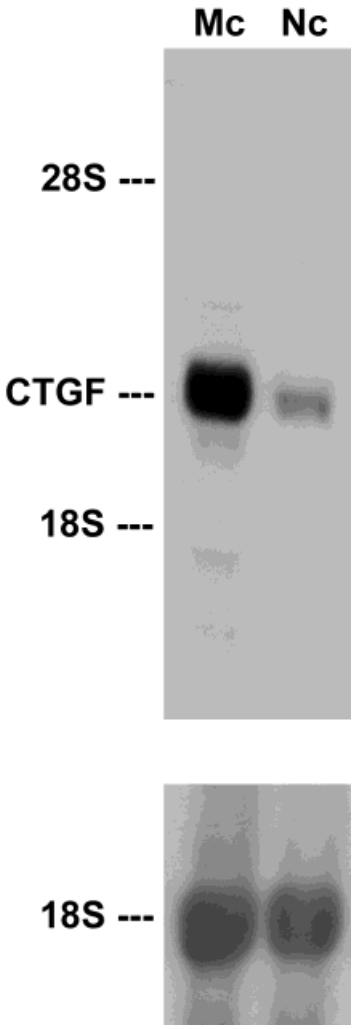

Fig. 2. Northern blot analysis of CTGF expression in bone. A total of 20 μg of RNA isolated from mutant (Mc) and normal (Nc) calvaria was loaded in each lane, electrophoresed, blotted, and probed for CTGF. The location of 28S and 18S rRNA is also indicated as determined from the ethidium bromide-stained gel. The blot was stripped and re-probed for 18S to serve as a control. Northern blot analysis was repeated three times using independent RNA samples and CTGF expression was between 8- to 10-fold higher in mutant versus normal bone in each case.

assembled from the overlapping RACE clones, PCR primers were designed based on this sequence, and the full-length rat CTGF cDNA was amplified from RNA isolated from *op* mutant rat calvaria. This clone was completely sequenced and was identical to our original consensus sequence (deposited in GenBank under accession number AF120275). The cloned rat CTGF has an open reading frame of 1,041 bp with 224 bp of 5′-untranslated sequence and 1,064 bp of 3′-untranslated sequence before the poly A tail (Fig. 3). The open reading frame encodes a protein of 347 amino acids with a predicted molecular weight of 37,753 daltons. The sequences surrounding the first ATG in this open reading frame (GCCCCGACCATGC) closely match (10/13-bp identity) the Kozak consensus sequence (GCCGCCA/GCCATGG) for

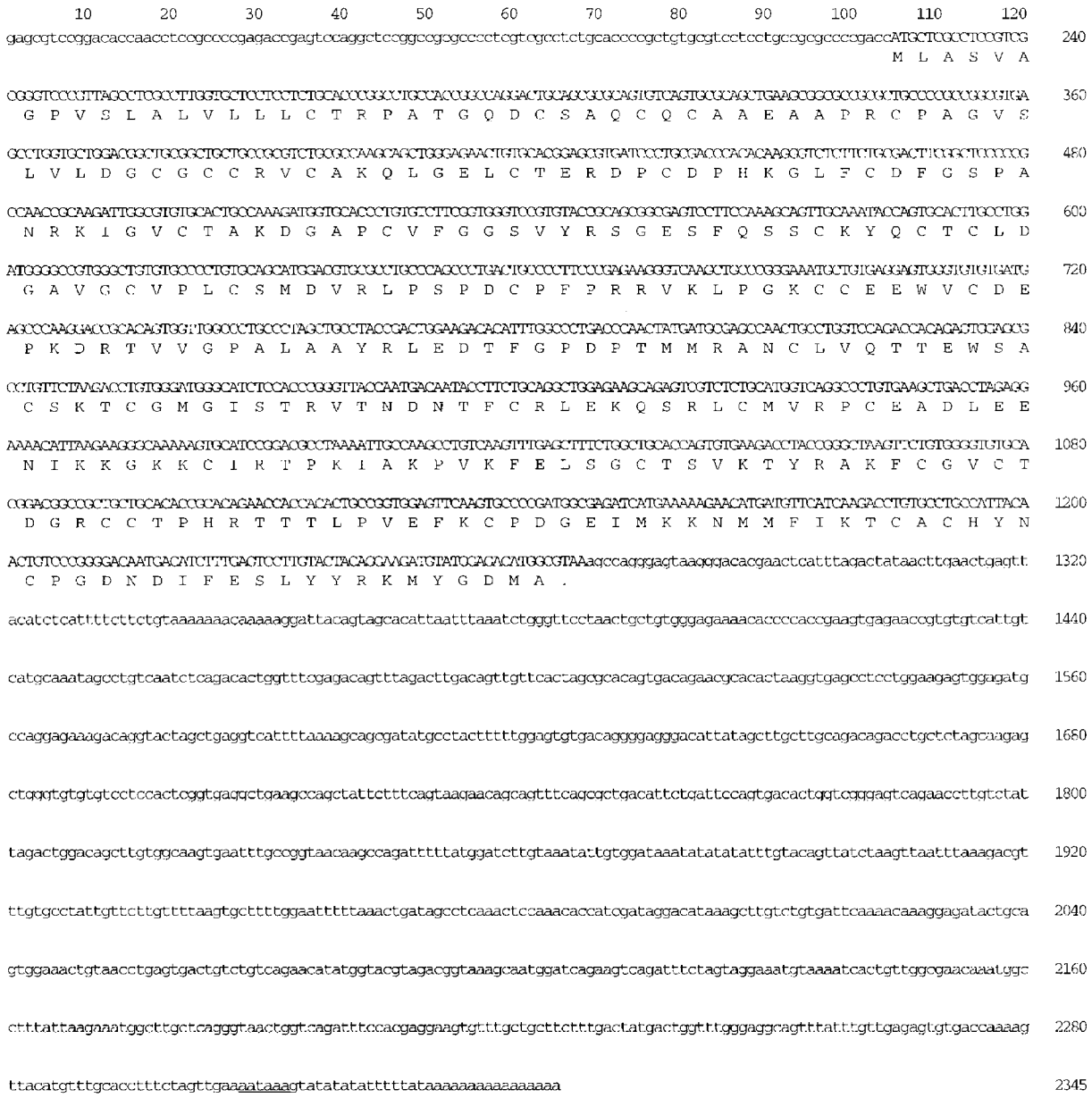

Fig. 3. Nucleotide and predicted protein sequence of rat CTGF. The open reading frame beginning at the initiation methionine at nucleotide 225 and encoding a protein with high homology to those encoded by the reported human, murine, porcine, and bovine CTGF cDNA is shown in capital letters. The predicted amino acid sequence of the encoded protein is shown in single-letter format below. A consensus polyadenylation signal (AATAAA) is underlined in the 3′ untranslated region of the sequence at base pair 2308.

initiation of translation [Kozak, 1989]. Underlined at position 2308 in the 3′-untranslated sequence in Figure 3 is a consensus polyadenylation signal (AATAAA). As the rat CTGF gene has not been characterized, it cannot be concluded that the 5′ end of our cDNA represents the transcriptional initiation site, although the total length (2,345 bp) is approximately the same as the size of the transcript observed in bone by Northern blot. As in most other tissues reported, only this single ~2.4-kb transcript was observed in bone.

Comparison of the sequence of the rat CTGF open reading frame (ORF) with the sequences reported for human [Bradham et al., 1991] and mouse [Brunner et al., 1991] CTGF revealed a highly conserved DNA sequence with 88% and 95% identity, respectively (Fig. 4A). The rat CTGF ORF was also found to be highly similar to the sequences reported for porcine (88% identical) [Brigstock et al., 1997] and bovine (85% identical) [Lin et al., 1998] CTGF (data not shown). A similarly high degree of conservation was also found at the amino acid level when the sequences of the putative CTGF proteins, derived from the DNA sequences of the open reading frames, were compared. As can be seen in Figure 4B, comparison of the putative rat and human CTGF proteins showed 91% identity while comparison of the rat and mouse CTGF proteins showed a 95% identity. The rat CTGF protein also showed 93% and 88% identity to the porcine and bovine CTGF proteins, respectively (data not shown). The greatest sequence divergence was apparent between the N-terminal domains of the proteins, a region that is predicted to be the signal sequence for secretion [Nielson et al., 1997]. The most probable cleavage site for the signal peptide is conserved in all reported mammalian CTGF proteins and is marked by the arrow in Figure 4B. Unlike the human CTGF protein, no potential sites of N-linked glycosylation are evident in the sequence of rat CTGF.

Northern blot analysis of multiple tissues from *op* mutant and normal rats showed that variable levels of CTGF expression were detectable in the thymus, liver, spleen, kidney and brain with highest levels in the latter two (Fig. 5). Interestingly, CTGF was also overexpressed in *op* compared with normal kidney (2- to 4-fold) with similar levels of expression observed between mutant and normal animals in all other tissues examined.

Primary rat osteoblast cultures were examined for CTGF expression at various stages of differentiation and in response to chronic treatment with PGE_2 over a period of 25 days. Figure 6 shows that CTGF expression is inversely related to the stage of osteoblast differentiation. Focusing on the vehicle treated cultures (Fig. 6, lanes V), it can be seen that CTGF mRNA was detectable throughout the 25-day period of culture. The highest levels of expression were during the early stages of osteoblast differentiation (days 8 and 12), with a progressive decrease in CTGF mRNA expression as the cells became terminally differentiated (as determined by the increasing expression of the bone phenotype markers, alkaline phosphatase (AP) and osteocalcin (OC) on days 18–25 of culture). Chronic treatment of the cultures with 10^{-6} M PGE_2 with each media replacement every 48 h resulted in significant upregulation of CTGF expression at 12, 18, 20, and 25 days of culture, with the magnitude of the response greatest in the older cultures. This treatment completely blocked differentiation, as demonstrated by the lack of induction of the osteoblast phenotypic markers, alkaline phosphatase and osteocalcin. This finding suggests that CTGF expression may be linked to the establishment and maintenance of the early osteoblast phenotype.

DISCUSSION

In this study, the technique of mRNA differential display was used to compare the expression of genes in normal versus osteopetrotic bone. Among the many differentially expressed genes, we isolated and cloned rat CTGF which is highly (8 to 10-fold) over-expressed in bone from *op* mutants compared with their normal littermates. Although the sequence for CTGF has been published in several species [Bradham et al., 1991; Ryseck et al., 1991; Brunner et al., 1991; Lin et al., 1998; Brigstock et al., 1997; Ying and King, 1996], this is the first report of the full-length CTGF coding sequence in the rat. Northern blot analysis of rat tissues other than bone showed that CTGF is expressed in the kidney, brain, thymus, spleen, and liver, with the highest levels of expression in the brain and kidney. Interestingly, CTGF expression was also upregulated (2- to 4-fold) in *op* versus normal kidney, but there were no significant differences between mutant and normal rats for any of the other tissues examined. Primary rat osteoblast cultures were examined

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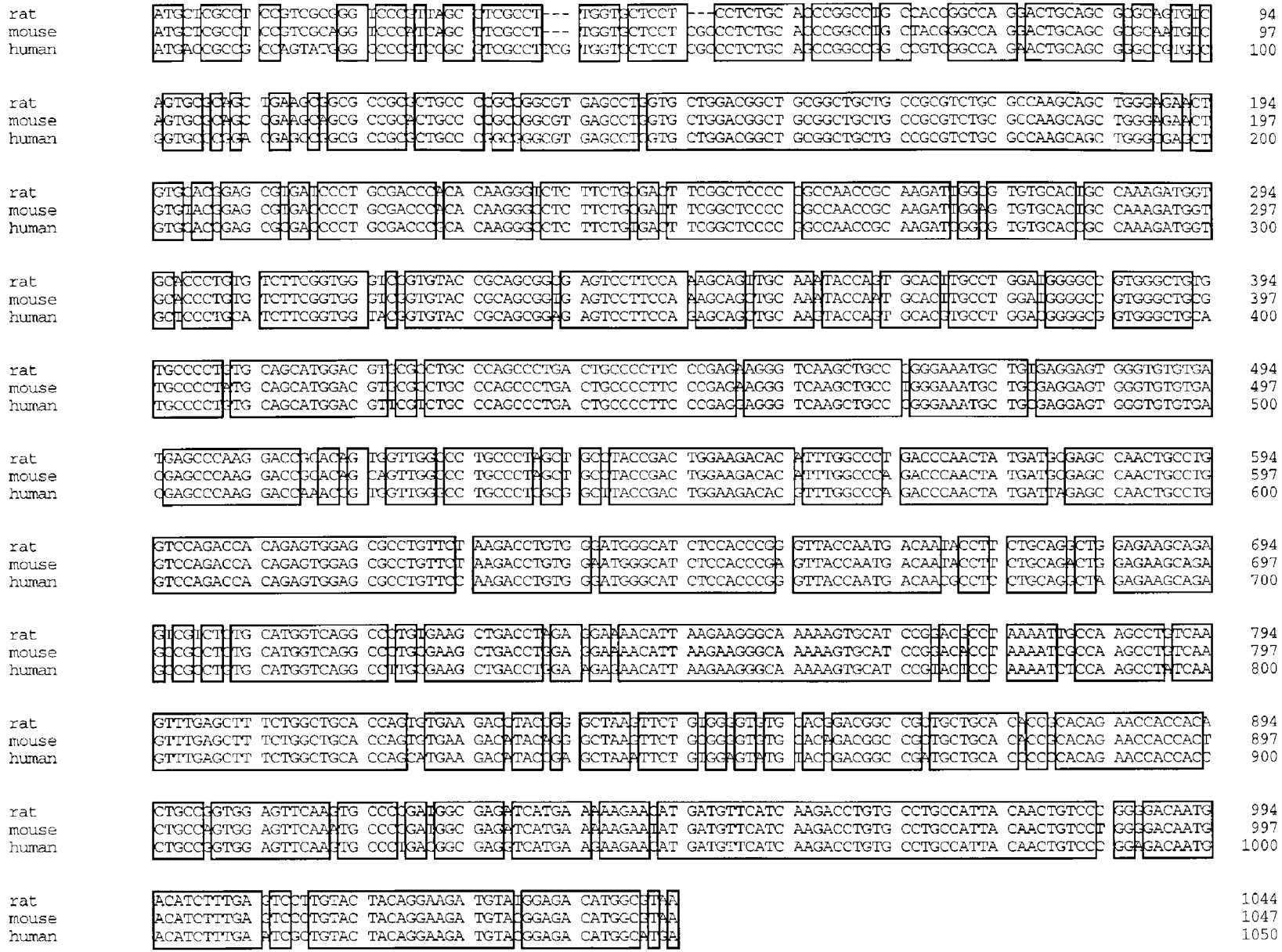
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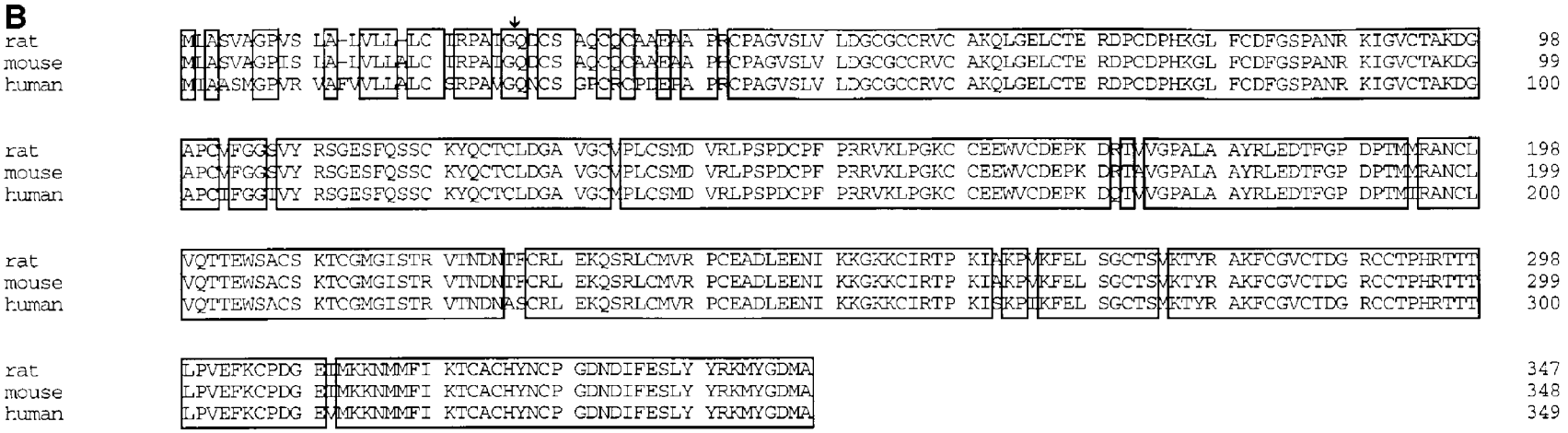

Fig. 4. cDNA (**A**) and amino acid (**B**) alignment of rat, mouse, and human CTGF sequences. **A:** The DNA sequences encoding the open reading frames of rat, mouse, and human CTGF are compared. Nucleotides conserved among the three species are boxed. Gaps have been introduced where necessary for alignment. **B:** The amino acid sequences deduced from the open frames of rat, mouse, and human CTGF are compared. Residues conserved among all three species are boxed. The most probable site of signal peptide cleavage is indicated by the arrow after residue 24 in the rat CTGF sequence.

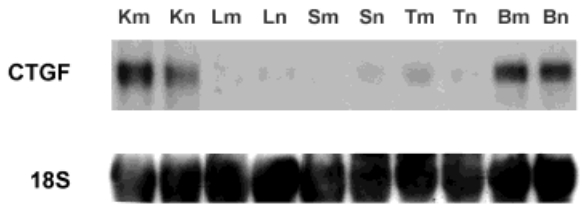

Fig. 5. Northern blot analysis of CTGF expression in rat tissues. Total RNA was isolated from mutant (m) and normal (n) tissues including kidney (K), liver (L), spleen (S), thymus (T), and brain (B). A total of 20 μg of RNA was loaded in each lane, electrophoresed, blotted and probed for CTGF. CTGF was detected in all tissues examined with highest levels of expression in brain and kidney; note that CTGF was overexpressed in mutant kidney, but similar to normal in all other tissues. Rehybridization with an 18S rRNA probe served as a control. Similar results were obtained in three separate experiments.

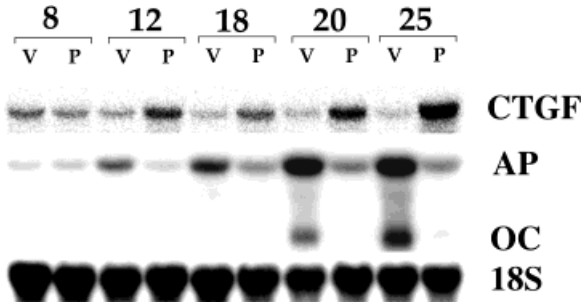

Fig. 6. Northern blot analysis of CTGF expression in primary osteoblast cultures. Total RNA was isolated from osteoblast cultures treated with 10^{-6} M PGE_2 (P) or vehicle (V) at 8, 12, 18, 20, and 25 days of culture. A total of 20 μg of RNA was loaded in each lane, electrophoresed, blotted and probed for CTGF. In the control (vehicle-treated) cultures, CTGF expression was highest at 8 days and progressively decreased thereafter persisting throughout the culture period. PGE_2 treatment upregulated CTGF expression with the magnitude of this effect being greatest at 20 and 25 days in culture. The blot was stripped and re-probed simultaneously with the osteoblast phenotype markers, alkaline phosphatase and osteocalcin, and then stripped and re-probed for 18S rRNA to normalize for differences in loading and/or blotting. This Northern blot analysis was repeated three times with similar results.

for CTGF mRNA expression at various stages of proliferation/differentiation demonstrating that CTGF was expressed at all stages, with the highest levels at earlier stages and a progressive decrease as the cells terminally differentiated. In addition, CTGF expression in these cultures was upregulated by chronic high-dose PGE_2 treatment, which effectively blocks differentiation. These data suggest that this growth factor may play a physiological role in the commitment/differentiation of osteoblasts. We have not yet investigated acute treatment of the rat osteoblast cultures with PGE_2 or other potential bone anabolic agents (i.e., PTH) to see whether CTGF mRNA expression may be directly regulated by such agents, or whether the changes in CTGF expression are an indirect effect of a PGE_2-induced blockade of osteoblast differentiation.

Differential display (DD) was first described as a way to bring the remarkable power and sensitivity of the PCR to bear on questions of differences in gene expression [Liang and Pardee, 1992; Liang et al., 1993]. The advantages of DD include its lack of bias and high sensitivity. The absence of bias means that no prior assumptions need to be made as to biological mechanisms, and the method is capable of detecting novel gene products. The main disadvantages of DD are that it is labor-intensive and susceptible to producing false-positive results. These can be minimized by attention to experimental design and other technical considerations. Our experimental approach included the use of inbred animal stocks to eliminate simple allelic polymorphisms and the pooling of samples from the same genotype to minimize individual differences in gene expression. Moreover, the tissue used as the RNA source in these experiments was critical. We used two independent sites, calvaria and long bone, to isolate RNA used as a template for DD and focused our attention on mRNA that were differentially expressed at both sites.

The utility of this approach in studies of bone biology is evidenced by the recent success of Mason and colleagues [1997] in applying DD to study bone cell gene expression. Using DD as their initial screening method, they made the unanticipated discovery that the glutamate signaling pathway used by the central nervous system appears to be involved in the response of bone to mechanical loading. Using the DD approach in this study, we isolated and cloned the rat CTGF cDNA and confirmed that it was highly over-expressed in *op* mutant versus normal bone. It will be important to verify that the level of CTGF protein in *op* tissues reflects the overexpression seen for the mRNA.

The rat mutation, *osteopetrosis* (*op*) was first described by Moutier and colleagues [1974]. Like *toothless* (*tl*) and *incisors-absent* (*ia*), *op* is an independent autosomal recessive mutation [Moutier et al., 1976] with characteristic phenotypic manifestations due to a failure of normal osteoclast function, i.e., a sclerotic skeleton generally lacking in marrow cavities. In *op* mu-

tants the sclerosis does not resolve with age and intertrabecular spaces become progressively more fibrotic [Marks and Popoff, 1989]. The skeletal sclerosis can, however, be cured by infusions of neonatal spleen or bone marrow cells from normal littermates and, conversely, osteopetrosis can be induced in normal littermates by infusion of these cells from *op* mutants [Popoff et al., 1994]. These findings are consistent with the hypothesis that the primary defect is autonomous to cells of the osteoclast lineage. However, abnormalities involving osteoblasts [Shalhoub et al., 1991] and mineral homeostasis [Hermey et al., 1995] have also been reported in *op* mutants. These include upregulation of several osteoblast-related genes such as alkaline phosphatase, osteopontin, osteocalcin, and fibronectin [Shalhoub et al., 1991]. Although these abnormalities are likely to be secondary to the underlying defect in osteoclast development/function, they are an important component of the pathophysiological changes that occur during bone development in *op* mutants.

It is known that bone formation, which includes the regulation of osteoblast differentiation, is tightly coupled to bone resorption. In osteopetrosis, this regulatory mechanism is dysfunctional which sets the stage for abnormal osteoblast proliferation/differentiation and function. In primary osteoblast cultures, we have shown that CTGF expression is highest during early stages when the cells are actively proliferating. As proliferation decreases and mineralized bone nodules and markers characteristic of the bone phenotype become evident, CTGF expression decreases. PGE_2 has been shown to increase proliferation and commitment of cells of the osteoblast lineage [Pilbeam et al., 1996], yet chronic PGE_2 exposure prevents their terminal differentiation. The inverse relationship between osteoblast differentiation and CTGF expression is a correlation suggesting that CTGF may play a role in the establishment and/or maintenance of the early osteoblast phenotype. A recent study by Kumar and colleagues [1999] showed that recombinant human CTGF-like protein (approximately 60% identity with CTGF) promotes the adhesion of osteoblasts and inhibits osteocalcin production in rat osteoblast-like ROS17/2.8 cells. Although our study does not provide direct evidence for an effect of CTGF on osteoblasts, studies are currently under way to examine the effects of recombinant rat CTGF on primary osteoblast cultures as well as bone formation in vivo.

Our findings suggest that CTGF plays a role in normal osteoblast differentiation and its overexpression in *op* bone may, at least in part, reflect abnormal osteoblast development (e.g., inhibition of terminal differentiation or an increase in the number of cells in the proliferation/commitment stage) in this mutation. Given the upregulation of CTGF in fibrotic soft tissues, where collagen is a major component [Ito et al., 1998; Dammeier et al., 1998; Oemar et al., 1997; Igarashi et al., 1996], it is perhaps not surprising to find CTGF over-expressed in the sclerotic, collagen-rich mineralized skeletal tissues of *op* rats. Whether CTGF is over-expressed in all osteopetrotic conditions, and as such could be a marker for them, should be investigated. Studies are currently under way to localize CTGF in both *op* and normal bone using in situ hybridization and immunohistochemical approaches.

The absence of normal hematopoietic and immune cells in the microenvironment of *op* bone is likely to have adverse effects on the local regulation of bone cell development and function. Although little is known about the expression of paracrine factors (cytokines) that affect bone cells in *op* mutants, a previous study showed that TGF-β expression is upregulated in *op* versus normal bone [Shalhoub et al., 1996], an observation that has been confirmed in our laboratory. Since other studies have clearly shown that CTGF expression is strongly induced by TGF-β [Grotendorst, 1997], the serine/threonine kinase signaling pathway mediated by activation of the TGF-β receptor may induce the increased CTGF expression in *op* bone. Based on previous studies showing that CTGF is coordinately expressed with TGF-β in several fibrotic disorders [Grotendorst, 1997], we postulate that this same mechanism may be involved in the pathogenesis of the intertrabecular fibrosis that is characteristic of this osteopetrotic mutation. Another possible mechanism may be related to the significant over-expression of the c-*fos* proto-oncogene in *op* bone [Safadi and Popoff, unpublished observation]. Previous studies characterizing the human and mouse CTGF gene promoter regions revealed multiple consensus elements characteristic of other serum- or growth factor-inducible genes including two AP-1 binding sites [Grotendorst, 1997]. These findings support the possibility

that c-*fos* may also play a role in upregulating the expression of CTGF in *op* bone. Clearly additional studies are necessary to determine the mechanism(s) that mediate CTGF expression in normal bone cells as well as its overexpression in *op* bone.

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